EXHIBIT 4

Telomerized human multipotent mesenchymal cells can differentiate into hematopoietic and cobblestone area–supporting cells

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Objective. Our objective was to compare the hematopoietic support provided by telomerized human mesenchymal stem cells (MSCs) and telomerized MSC-derived stromal cells.

Methods. We transfected the human telomerase catalytic subunit (hTERT) gene into primary MSCs to establish hTERT-transduced MSCs (hTERT-MSCs). Stromal induction of hTERT-MSCs was performed by replacing the culture medium with Dexter-type culture medium. Hematopoietic support was examined by coculture with cord blood CD34^{+} cells.

Results. The hTERT-MSCs were morphologically identical with the primary MSCs and expressed surface antigens including CD105, CD73, and CD166. hTERT-MSCs showed a similar doubling time as primary MSCs and continued to proliferate to over 80 population doublings (PD), although the primary MSCs underwent crisis in vitro at 16 PD. The osteogenic, chondrogenic, adipogenic, neurogenic, and stromal differentiation potential of hTERT-MSCs were maintained up to at least 40 PD. The degree of expansion of CD34^{+} cells and total number of colony-forming units in culture (CFU-C) upon 12-day coculture with the hTERT-MSC–derived stromal cells were nearly the same as those upon 12-day coculture with hTERT-MSCs (CD34, 33.0-fold ± 2.8-fold vs 36.1-fold ± 1.7-fold of the initial cell number; CFUs, 344.4-fold ± 62.5-fold vs 239.3-fold ± 87.0-fold; CFU-mix, 368.4-fold ± 113.7-fold vs 341.3-fold ± 234.3-fold). However, on day 18 of coculture, the number of cobblestone areas (CA) observed beneath the stromal cells was 15 times higher than that beneath hTERT-MSCs (CA, 146.9 ± 54.6 vs 9.4 ± 8.1, $p < 0.01$).

Conclusion. Stromal induction of hTERT-MSCs exclusively enhanced the support of CA formation provided by hTERT-MSCs. Our human hTERT-MSCs will be useful for elucidating the mechanism of the formation of CAs. © 2003 International Society for Experimental Hematology. Published by Elsevier Inc.

Mesenchymal stem cells (MSCs) are multipotent and self-renewing, and can repopulate the tissue in which they reside [1–3]. Cells endowed with these properties have been isolated from several tissues including bone marrow (BM) [4–9], umbilical cord blood (CB) [10,11], skin [12], and adipose tissue. Among them, attention has been paid to BM-derived MSCs (BM-MSCs) because they are easier to obtain than

MSCs from other sources, and they differentiate into a variety of tissues in addition to osteogenic tissue of the BM such as adipocytes, chondrocytes, and osteocytes under a special culture condition or following transplantation [13–19].

In addition to renewing osteogenic tissue, BM-MSCs contribute to the formation of the BM microenvironment that supports hematopoiesis including stromal cells, and recent findings suggested that BM-MSCs not only support the proliferation of hematopoietic progenitor cells but also play a role distinct from stromal cells in the support of

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hematopoiesis [20–23]. However, the possibilities that a different property of the cells or clones was selected during the establishment of the primary MSCs and/or stromal cells in previous studies, and that the aging and senescence of primary human cells in in vitro culture were being utilized, cannot be ruled out [8,24]. To overcome these problems, establishment of human BM-MSC lines that maintain the potential of stromal differentiation and the ability of hematopoietic support should be useful in obtaining stromal cells that are identical with their parental MSCs.

Recently, Shi [25] and Simonsen et al. [24] demonstrated that human telomerase catalytic subunit (hTERT) gene transduction prolonged the lifespan of osteogenic human BM-MSCs. More recently, we demonstrated that human BM hematopoietic-supporting stromal cells transduced with the hTERT gene showed prolonged lifespan in the Dexter culture condition [26]. However, it is not clear whether hTERT-transduced MSCs maintain their potential of stromal differentiation and ability of hematopoietic support.

In the present study, we established human hTERT-transduced MSCs (hTERT-MSCs) and induced stromal differentiation of the hTERT-MSCs. We compared the hematopoietic support provided by the multipotent hTERT-MSCs and the hTERT-MSC–derived stromal cells.

Materials and methods

Long-term BM-MSC cultures

Human BM was obtained by aspiration from the posterior iliac crest of three healthy adult volunteers after informed consent was obtained. This study was approved by the Institutional Review Board at our university. BM mononuclear cells (MNCs) were plated in 150-cm^2 plastic tissue culture flasks and incubated overnight. After washing out the free cells, the adherent cells were cultured in Dulbecco's modified essential medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL, Rockville, MD, USA) at 37°C under 5% CO_2 in a humidified atmosphere. After reaching confluence, they were harvested and cryopreserved as primary MSCs or used for gene transduction.

Retroviral vector and transduction of BM-MSCs

The retroviral vector, BABE-hygro-hTERT, was employed in these experiments. Amphotropic viral supernatant containing BABE-hygro-hTERT was generated by packaging cell line ψCRIP-P131 as previously described [26]. Two hundred thousand primary MSCs in a 10-cm dish were exposed to viral supernatant containing retrovirus at an approximate multiplicity of infection of one to ensure single-copy integration, in the presence of 8 μg/mL polybrene (Sigma, St. Louis, MO, USA) for 8 hours. After washing with phosphate-buffered saline (PBS), the transduced MSCs were selected with 0.1 mg/mL hygromycin.

Analysis of telomerase activity and karyotype

The level of telomerase activity in the primary MSCs and hTERT-MSCs was analyzed by the stretch polymerase chain reaction (PCR) method using a TeloChaser (TOYOBO, Osaka, Japan) as described previously [26,27]. The amplified telomeric repeats were then separated by electrophoresis in a 10% polyacrylamide gel and visualized by staining with SYBR GREEN I (Molecular Probes, Eugene, OR, USA). Karyotyping was performed according to the International System for Human Cytogenetic Nomenclature [28].

Evaluation of the differentiation potential of human MSCs

The differentiation potential of hTERT-MSCs into osteocytes, adipocytes, or chondrocytes was studied using differentiation-induction media purchased from Bio-Whittaker (Walkersville, MD, USA) according to provided protocols. Briefly, to induce osteogenic differentiation, primary MSCs or hTERT-MSCs were seeded at a density of 3×10^3 cells/cm^2 in tissue culture dishes and cultured with MSC growth medium containing 100 nM dexamethasone, 50 μM ascorbic acid 2-phosphate, and 10 mM β-glycerophosphate. The cultures were maintained for 3 weeks and the culture medium was replaced every 3 days. The osteogenic induction of cells was confirmed by alkaline phosphatase staining.

To induce adipogenic differentiation, primary MSCs or hTERT-MSCs were seeded at a density of 2×10^4 cells/cm^2 in tissue culture dishes. When the MSCs reached confluence, adipogenic differentiation was maintained by the induction medium that contained DMEM with 10% FBS, 1 μM dexamethasone, 0.2 mM indomethacin, 10 μg/mL insulin, and 0.5 mM 3-isobutyl-1-methylxanthine. Cells were rinsed twice with PBS and fixed with 10% buffered formalin at room temperature for 10 minutes. Fixed cells were stained with oil-red to confirm lipid deposition.

To induce chondrogenic differentiation, 2.5×10^5 primary MSCs or hTERT-MSCs were placed in a 15-mL polypropylene tube. After centrifugation at 150g at room temperature for 5 minutes, the cells were resuspended in 0.5 mL of high-glucose DMEM supplemented with 10 ng/mL of recombinant human TGF-β3, 100 nM dexamethasone, 6 μg/mL insulin, 100 μM ascorbic acid 2-phosphate, 1 mM sodium pyruvate, 6 μg/mL transferrin, 0.35 mM proline, and 1.25 mg/mL bovine serum albumin (BSA). The MSCs were centrifuged again at 150g at room temperature for 5 minutes and maintained as a small pellet for 21 days. The pellet was embedded in paraffin and sections of 10-μm thickness were stained with Alcian blue.

Neuronal induction was performed by the procedure reported by Woodbury et al. [17]. When the hTERT-MSCs reached subconfluence, the culture medium was replaced with preinduction medium consisting of DMEM with 20% FBS and 1 mM β-mercaptoethanol (BME) (Sigma Chemical Corp., St. Louis, MO, USA), 24 hours prior to neuronal induction. To induce neuronal differentiation, the preinduction medium was removed and the cells were transferred to neuronal induction medium, which was composed of DMEM with 2% dimethylsulfoxide (Sigma Chemical Corp., St. Louis, MO, USA) and 200 μM butylated hydroxyanisole. Cells were fixed, and immunohistochemical staining was performed using anti-human neuron-specific enolase (NSE) monoclonal antibody as described below.

Stromal differentiation was induced by replacing the culture medium with Dexter-type (DT) long-term bone marrow culture medium (LTCM), which was composed of minimum essential medium-α, 12.5% horse serum (Gibco BRL, Rockville, MD, USA), 12.5% fetal calf serum (Gibco BRL), 1×10^{-6} M hydrocortisone (Sigma Chemical Corp., St. Louis, MO, USA), and 1×10^{-4} M BME [15,29]. The cells were incubated at 37°C under 5% CO_2 in a humidified atmosphere for more than 10 days.

Immunohistochemical staining

Forty thousand hTERT-MSCs were plated on Type I collagen-coated culture slides (BIOCOAT; Becton-Dickinson, Bedford, MA,

USA) 24 hours before placing the cells in the neuronal- or stromal-induction medium. Some culture slides were incubated without placement in an induction medium as a negative control. After neuronal induction, the cells were washed by PBS three times and fixed with cold methanol at 4°C for 30 minutes. After stromal induction, the cells were washed by PBS three times and fixed with 3.7%(v/v) formaldehyde in PBS at 4°C for 30 minutes. Cells were permeabilized by 0.2% (v/v) Triton X-100 in PBS at 4°C for 5 minutes. Immunostaining was conducted using the VECTASTAIN ABC Kit (Burlingame, CA, USA) according to the manufacturer's instructions. Anti-alpha-smooth muscle actin (ASMA) (Clone 1A4, DAKO) monoclonal antibody (mAb), anti-NSE (Pharmingen, San Diego, CA, USA) mAb, or isotype control (Chemicon, Temecula, CA, USA) was utilized as the primary antibody, and rhodamine-conjugated anti-mouse IgG (DAKO, Glostrup, Denmark) or biotin-ylated anti-mouse IgG (Funakoshi, Tokyo, Japan) was employed as the secondary antibody. Cells were visualized under a confocal microscope (ZEISS/PASCAL, Munich, Germany) or by utilizing the DAB kit (Funakoshi, Tokyo, Japan).

Coculture of hematopoietic cells with hTERT-MSCs or stromal cells

Thirty thousand hTERT-MSCs were plated in 25-cm^2 plates. On the next day, the culture medium in half of the plates was replaced by DT-LTCM for induction into stromal cells. When the hTERT-MSCs and hTERT-MSC–derived stromal cells reached over 90% confluence, the cells were washed five times with PBS before the addition of CB CD34$^+$ cells (Takara, Tokyo, Japan). Five thousand CB CD34$^+$ cells were seeded on a monolayer of hTERT-MSCs or hTERT-MSC–derived stromal cells that had been preestablished in 5 mL of a serum-free medium, X-VIVO 10 (Bio Whittaker, Walkersville, MD, USA), supplemented with 50 ng/mL human thrombopoietin (TPO; a gift from Kirin Brewery Co. Ltd., Tokyo, Japan), 10 ng/mL human stem cell factor (SCF; provided by Kirin Brewery), and 50 ng/mL human Flk-2/Flt-3 ligand (FL; R&D Systems, Minneapolis, MN, USA) at 37°C under 5% CO$_2$. After 5 days of coculture, 5 mL of fresh complete medium containing the same concentrations of cytokines was added and the coculture was continued for 7 days. At the end of 12 days of coculture, nonadherent and adherent hematopoietic cells that were weakly attached to stromal cells were collected by gentle pipetting. The phenotype of the expanded hematopoietic cells was analyzed as described below. The coculture was maintained by adding 5 mL of fresh medium containing cytokines on day 12 in order to observe the formation of cobblestone areas (CAs) from adherent hematopoietic cells and hematopoietic cells that transmigrated beneath the layer of stromal cells.

Immunophenotyping of hTERT-MSCs and ex vivo–expanded hematopoietic cells

Flow cytometric analysis of MSCs and ex vivo–expanded hematopoietic cells was performed as previously described [26,30]. Briefly, cell suspensions were washed twice with PBS containing 0.1% BSA. The cells were blocked in PBS with 10% normal mouse serum (Pharmingen, San Diego, CA, USA) at 4°C for 10 minutes prior to staining. For direct assays, one million cells were incubated with FITC-conjugated CD34, CD45, CD9 (Immunotech, Marseille, France), CD166 (ALCAM) (Antigenix America, Huntington, NY, USA), CD105 (SH-2), CD106 (Ancell, Bayport, MN, USA), or

CD157 (MBL, Nagoya, Japan) and PE-conjugated CD34, CD38, CD41, CD90, glycophorin A, CD117 (Immunotech), CD19, CD11b, CD3 (Dako Japan, Kyoto, Japan), or CD133 (Miltenyi Biotech, Auburn, CA) at 4°C for 30 minutes, and then washed twice with PBS containing 0.1% BSA. For indirect assays, cells were immunolabeled with anti-human CD73 (SH-3; Alexis Biochemicals, San Diego, CA, USA). As the secondary antibody, goat anti-mouse IgG (H + L)-FITC (Immunotech) was used. The cells were analyzed by flow cytometric analysis using an EPICS XL flow cytometer (Becton-Dickinson, San Diego, CA, USA) with EXPO32 software.

Evaluation of clonogenic cells and formation of CA

The clonogenic assay was performed as previously described [26]. Aliquots of hematopoietic cells obtained from 12-day coculture were cultured in quadruplicate using 35-mm tissue culture dishes in 1 mL of 0.9% methylcellulose medium containing 30% fetal calf serum, 1% BSA, 50 U/mL penicillin, 50 μg/mL streptomycin, 2 mM L-glutamine, 1×10^{-4} M BME, 3 U/mL recombinant human erythropoietin, 10 ng/mL human interleukin (IL)-3, 50 ng/mL SCF, and 10 ng/mL human granulocyte-macrophage colony-stimulating factor (GM-CSF) (MethoCult GF H4434V, Stem Cell Technologies, Vancouver, BC, Canada). After 14 days of culture in a humidified environment at 37°C under 5% CO$_2$, colonies consisting of 50 or more cells were scored under a microscope. The number of CAs including more than 5 cells was counted on day 18 of coculture before they fused with each other.

Statistical Analyses

Results are expressed as the mean ± standard deviation (SD). The significance of differences was assessed by Mann-Whitney U-test. Significance was set at $p < 0.05$.

Results

Establishment of hTERT-MSCs

We compared the characteristics of the established hTERT-MSCs with those of the primary MSCs. The doubling time (DT) of the primary MSCs was approximately 5 days (Fig. 1A). The primary MSCs gradually flattened by PD = 16 and their growth arrested on approximately the 40th day of culture. The hTERT-MSCs grew at a similar rate (DT = 5 days) as the primary MSCs and continued to grow at the same rate after the primary MSCs ceased dividing. The hTERT-MSCs could be cultured beyond 400 days (PD = 80). The morphological characteristics of the primary MSCs at PD = 10 and that of the hTERT-MSCs at PD = 40 were similar (Fig. 1B); they both had organelle-rich cytoplasm with a few nucleoli. The osteogenic, adipogenic, chondrogenic, and neurogenic potential of the hTERT-MSCs at PD = 40 were maintained (Fig. 1B). These results suggested that hTERT transduction prolonged the lifespan of MSCs without losing their multipotentiality. Next, we assessed the level of telomerase activity in the primary and hTERT-MSCs by the stretch PCR method (Fig. 2A). Apparent telomerase activity was detected in the hTERT-MSCs at PD = 40,
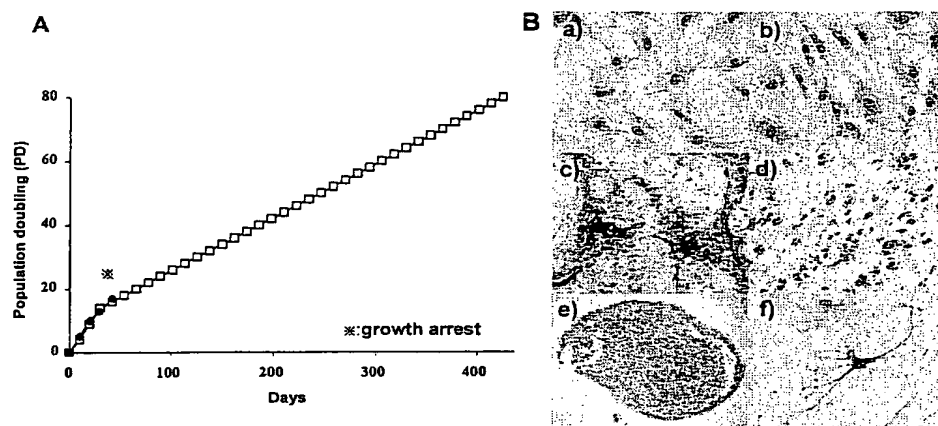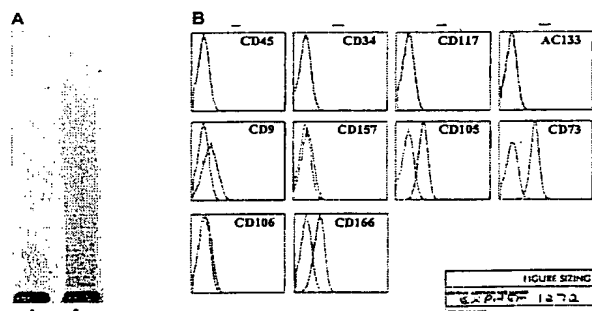
Figure 1. Population doubling of primary and hTERT gene-transduced human mesenchymal cells, morphology and multipotentiality. (A) The x-axis indicates the number of incubation days and the y-axis indicates the number of population doublings. ● = primary MSCs; □ = hTERT-MSCs. (B) (a) May-Giemsa staining of primary MSCs at PD = 10 (×100). (b) May-Giemsa staining of hTERT-MSCs at PD = 40 (×100). (c) ALP staining of hTERT-MSCs at PD = 40 (×100). (d) Oil-red staining of hTERT-MSCs at PD = 40 (×100). (e) Alcian blue staining of hTERT-MSCs at PD = 40 (×100). (f) Immunostaining of hTERT-MSCs at PD = 40 (×100) after differentiation in the neuronal induction medium by anti-human NSE.

whereas it was not detectable in the primary MSCs at PD = 10.
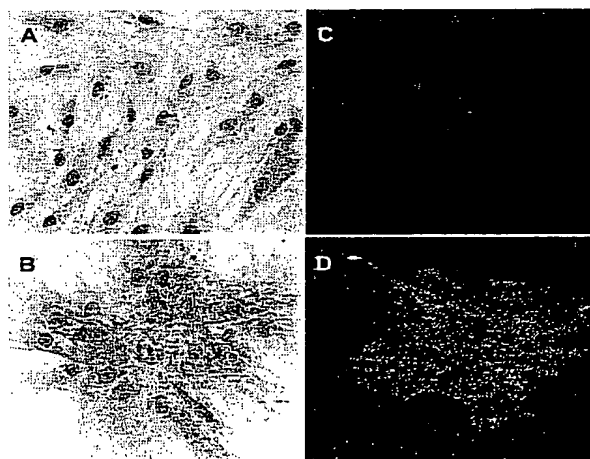
Surface antigens of the hTERT-MSCs

The surface antigens on hTERT-MSCs at PD = 40 were analyzed by flow cytometry. The hTERT-MSCs expressed CD9, CD166, CD105, and CD73 (Fig. 2B), in agreement with the expression pattern of surface antigens on primary human MSCs that had been previously reported [31]. We also confirmed that the hTERT-MSCs at PD = 40 had a normal karyotype (data not shown).

Stromal differentiation of the hTERT-MSCs

Stromal differentiation was induced by replacing the culture medium. The hTERT-MSCs in DT-LTCM, but not in regular medium, grew with colony-like formation (Fig. 3). It was reported that stromal cells are mainly composed of myofibroblastic cells that had differentiated from mesenchymal cells, by following a vascular smooth-muscle lineage characterized by the expression of ASMA [29,32–34]. We therefore examined the expression of ASMA in hTERT-MSCs and

Figure 2. Telomerase activity and flow cytometric analysis of the expression of surface antigens. (A) The level of telomerase activity, as determined by the stretch PCR method, was used to assess expression of the hTERT transgene. Lane 1, Primary MSCs; Lane 2, hTERT-MSCs at PD = 40. Data shown are from one representative experiment of two showing similar results. (B) hTERT-MSCs at PD = 40 were immunolabeled with the FITC-conjugated monoclonal antibody specific for the indicated surface antigen. Dead cells were eliminated by forward and side scatter. Data shown are hTERT-MSCs at PD = 40 from one representative experiment of three showing similar results.

Figure 3. Differentiation of hTERT-MSCs into stromal cells. May-Giemsa staining of hTERT-MSCs (A) or stromal cells derived from hTERT-MSCs (B). Immunostaining of hTERT-MSCs (C) or stromal cells derived from hTERT-MSCs (D) by anti-ASMA. Immunolabeled cells were visualized as a confocal image.

stromal-induced hTERT-MSCs. Increased ASMA expression was observed in the hTERT-MSCs after stromal induction by DT-LTCM, while weak ASMA expression was sporadically detected in the parental hTERT-MSCs (Fig. 3C and D).

Hematopoietic support by
hTERT-MSCs and stromal cells in vitro

Recent studies revealed that BM-MSCs directly support the proliferation of hematopoietic progenitor cells [20,22]. Therefore, we compared the hematopoietic cell (HPC) support provided by primary or hTERT-MSCs with that provided by hTERT-MSC–derived stromal cells. CB CD34$^+$ cells were cultured in the following four conditions for 12 days: 1) in the presence of SCF, TPO, and FL without hTERT-MSCs or stromal cells; 2) in the presence of the three cytokines with primary MSCs; 3) in the presence of the three cytokines with hTERT-MSCs; or 4) in the presence of the three cytokines with stromal cells. Even in the absence of both hematopoietic-supporting cells, a low level of expansion of hematopoietic cells was observed, owing to the presence of the three cytokines. Conversely, as expected, the total number of cells, and number of CD34$^+$ cells, CFU-C (colony forming units in culture), and CFU-Mix (mixed colony-forming units) 12 days after the start of coculture with primary hTERT-MSCs or stromal cells, were remarkably increased in comparison with the initial number of the respective cells (Table 1), suggesting that the hTERT-MSCs could support the hematopoietic progenitor cells to a similar degree as the primary MSCs and hTERT-MSC–derived stromal cells during this period. Since cytokines were added at relatively high concentration, they might explain the lack

of significant difference for nucleated cells and CFU between MSCs and stroma derived from MSCs. The expansion of hematopoietic cells in the absence of the three cytokines, when cultured with hematopoietic-supporting cells, was background level.

Because hematopoietic cells were observed sporadically beneath the stromal cell layer as well as beneath the hTERT-MSCs, the cocultures were continued by adding cytokines. At 18 days, the hematopoietic cells beneath the stromal cells had divided and exhibited a cobblestone appearance, while most of the hematopoietic cells under the layer of primary or hTERT-MSCs remained as single cells (Fig. 5A and B and Table 1). The number of CAs below stromal cells [AQ2] was 15 times higher than that below hTERT-MSCs (Table 1). In addition, the expression of lineage markers such as glycophorin A, CD11b, CD41, CD3, and CD19 was analyzed by flow cytometry. There were no significant differences in the expression levels of lineage markers on hematopoietic cells that had been cocultured with hTERT-MSCs or stromal cells (Fig. 4). These results suggest that hTERT-MSCs can support the expansion of hematopoietic progenitor cells and their differentiation to multiple lineages to a similar extent as stromal cells, and that the ability of CA support may be enhanced after induction into stromal cells.

Discussion

In the present study, we established a long-term culture of a human hTERT-transduced human MSC population by simple transduction of hTERT cDNA without a cloning procedure, and the potency of multilineage differentiation and the ability of hematopoietic support were maintained through PD = 40 (Fig. 1 and Table 1). In contrast, the primary MSCs gradually flattened and subsequently ceased to divide at PD = 16. Therefore, it is calculated that the hTERT-MSCs can divide 24 times without losing multipotency, and that we can obtain a 16 million-fold higher number of hTERT-MSCs than primary MSCs.

hTERT-transduced human fibroblasts [35], retinal pigment epithelial cells [36], and endothelial cells [37] were successfully cultured over the long term ex vivo, whereas hTERT-transduced human keratinocytes and epithelial cells could not be cultured over a long period of time unless there was reduced p16 expression [38]. Whether immortalization of primary human cells can be achieved by the ectopic expression of hTERT might depend on the cell type or the culture conditions. We previously reported that hematopoietic-supporting stromal cells transduced with the hTERT gene showed prolonged lifespan in the Dexter culture condition [26]. In the present study, we demonstrated that a human MSC population transduced with the hTERT gene could be cultured over the long term. However, these results are inconsistent with a recent report indicating that a human MSC clone could not be immortalized by transduction of the hTERT gene alone [39]. Immortalization of human MSCs

Table 1. Ex vivo expansion of CB CD34$^+$ cells for 12 days and the formation CAs

	Cytokines only	Primary MSCs	hTERT-MSCs	Stromal cells
Total cells	38.0 ± 3.8	474.7 ± 70.4*	368.5 ± 16.7*	408.5 ± 83.0*
CD34$^+$ cells	1.4 ± 0.1	38.5 ± 2.35*	36.1 ± 1.7*	33.0 ± 2.8*
CFU-C	12.6 ± 1.7	237.6 ± 65.4*	239.3 ± 87.0*	344.4 ± 62.5*
CFU-Mix	7.3 ± 3.7	254.2 ± 63.5*	341.3 ± 234.3*	368.4 ± 113.7*
CA	N.D.	7.3 ± 3.8	9.4 ± 8.1	146.9 ± 54.6†

Values of total cells and clonogenic cells indicate the fold increase at the end of the first period in comparison with the initial number of the respective cells. The number of CFU-C and CFU-mix before the culture were 1200 ± 235 and 62.5 ± 7.5, respectively, out of the 5000 CB CD34$^+$ cells.

*$p < 0.05$ vs cytokines only (n = 4). The values of CA indicate the absolute number of cobblestone areas which consisted of more than 5 cells originated from the initial 5000 CB cells, on day 18.

†$p < 0.01$, stromal cells vs primary of hTERT-MSCs. N.D. = not determined. Data shown are from one representative experiment of two showing similar results. The results are expressed as mean ± standard deviation (n = 4).
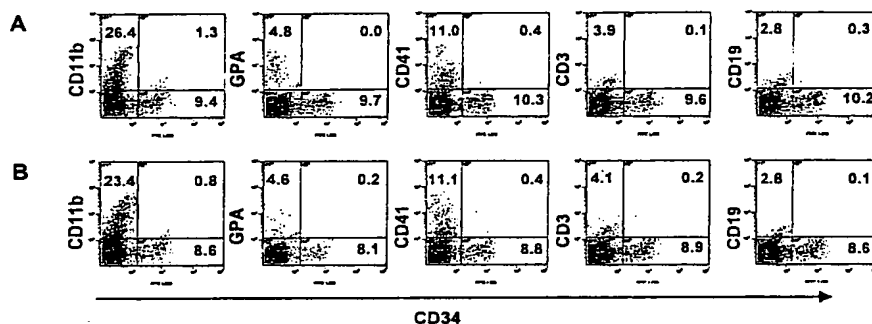
Figure 4. Expression of surface antigens on expanded hematopoietic cells. Expression of surface antigens on hematopoietic cells that had been generated after 12-day expansion of CB-derived CD34+ cells on hTERT-MSCs (A) or stromal cells (B). The x-axis indicates CD34 expression labeled with FITC-conjugated monoclonal antibody. The y-axis indicates CD11b, glycophorin A (GPA), CD41, CD3, or CD19 expression labeled with PE-conjugated monoclonal antibody. Cells were gated based on forward and side light scatter to exclude debris. Positivity for a surface antigen was defined using the isotype control monoclonal antibody. Data are from 2 independent experiments, each done in quadruplicate.
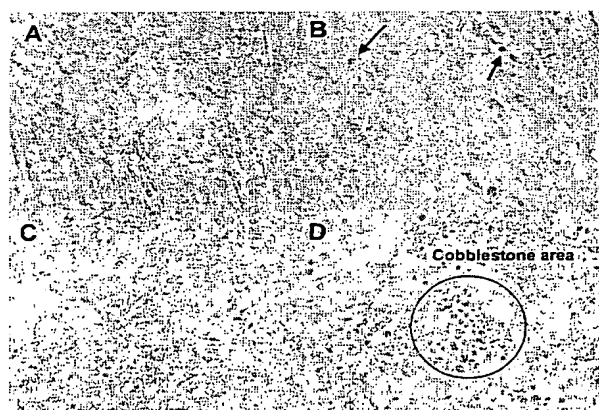
by the transduction of hTERT cDNA may require several clones or it may occur in a paracrine fashion in culture.

We also confirmed that the lifespan of MSCs derived from several individuals could be prolonged by transduction of the hTERT gene. This procedure may be useful for establishing a patient's own MSCs, and cells or tissues that had differentiated from them can be utilized for autologous transplantation in individual patients [40,41]. Furthermore, the hTERT-MSCs could be cryopreserved. Thus, we can easily propagate a large quantity of these human hTERT-MSCs.

In the present study, we attempted to induce the differentiation of hTERT-MSCs into stromal cells by replacing the culture medium with DT-LTCM, and obtained cells expressing higher levels of ASMA. It was reported that stromal cells are mainly composed of myofibroblastic cells that had

Figure 5. Formation of cobblestone areas beneath stromal cells derived from hTERT-MSCs. CD34+ cells were expanded for 18 days on hTERT-MSCs (A,B), or on stromal cells derived from hTERT-MSCs (C,D). Arrows indicate hematopoietic cells that transmigrated below hTERT-MSCs, and the circle indicates a cobblestone area (A, ×100; B, ×400; C, ×100; D, ×400).

differentiated from mesenchymal cells, by following a vascular smooth-muscle differentiation pathway characterized by the expression of ASMA [29,32–34]. Based on these reports, we employed these ASMA-expressing cells as stromal cells. Postnatal BM is known to contain both MSCs and stromal cells [42]. However, the difference in the level of hematopoietic support between these two types of cells has not been well understood [20–23]. Earlier reports suggested that primary MSCs supported CD34+ cells to a similar degree as primary stromal cells [20,21] and that human MSCs provide key signals to stimulate megakaryocyte and platelet production from CD34+ hematopoietic cells [20,22]. However, it was possible that a different property of the cells or clones was selected during the establishment of both the primary MSCs and stromal cells and that the aging of these primary cells was being utilized. To rule out this possibility, we set up a comparative experiment to evaluate the difference in hematopoietic support between the hTERT-MSCs and the stromal cells that had directly differentiated from the hTERT-MSCs. In this experiment, there was no detectable difference in the hematopoietic support provided by hTERT-MSCs and stromal cells in terms of the total number of cells, number of CD34 and clonogenic cells, and the percentage of lineage markers expressed on HPCs that had been cocultured with either hTERT-MSCs or stromal cells (Table 1 and Fig. 4). The only difference in hematopoietic support was the number of CAs that formed beneath the layer of hTERT-MSCs or hTERT-MSC–derived stromal cells. The hTERT-MSCs supported only a few CAs, while stromal cells supported multiple CAs, which have been reported to contain hematopoietic stem/progenitor cells [43,44] (Fig. 5 and Table 1). Thus, comparative analysis of the surface molecules expressed on hTERT-MSCs and hTERT-MSC–derived stromal cells may be useful to unravel the mechanism of the formation of CAs.

In conclusion, hTERT-MSCs exhibited a prolonged lifespan without losing multipotentiality. Upon stromal induction of hTERT-MSCs, the support of CAs provided by

hTERT-MSCs was exclusively elevated. Our human hTERT-MSCs will be useful for analyzing the change in hematopoietic support during differentiation of human MSCs to stromal cells.

References

1. Wakitani S, Saito T, Caplan AI. Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle Nerve. 1995;18:1417–1426.
2. Dennis JE, Caplan AI. Differentiation potential of conditionally immortalized mesenchymal progenitor cells from adult marrow of a H-2Kb-tsA58 transgenic mouse. J Cell Physiol. 1996;167:523–538.
3. Javazon EH, Colter DC, Schwarz EJ, Prockop DJ. Rat marrow stromal cells are more sensitive to plating density and expand more rapidly from single-cell–derived colonies than human marrow stromal cells. Stem Cells. 2001;19:219–225.
4. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284:143–147.
5. Jaiswal RK, Jaiswal N, Bruder SP, Mbalaviele G, Marshak DR, Pittenger M.F.. Adult human mesenchymal stem cell differentiation to the osteogenic or adipogenic lineage is regulated by mitogen-activated protein kinase. J Biol Chem. 2000;275:9645–9652.
6. Guo Z, Yang J, Liu X, et al. Biological features of mesenchymal stem cells from human bone marrow. Chin Med J (Engl). 2001;114:950–953.
7. Conget PA, Minguell JJ. Adenoviral-mediated gene transfer into ex vivo expanded human bone marrow mesenchymal progenitor cells. Exp Hematol. 2000;28:382–390.
8. Muraglia A, Cancedda R, Quarto R. Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. J Cell Sci. 2000;113(Pt 7):1161–1166.
9. Prockop DJ, Sekiya I, Colter DC. Isolation and characterization of rapidly self-renewing stem cells from cultures of human marrow stromal cells. Cytotherapy. 2001;3:393–396.
10. Erices A, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. Br J Haematol. 2000;109:235–242.
11. Barry F, Boynton R, Murphy M, Haynesworth S, Zaia J. The SH-3 and SH-4 antibodies recognize distinct epitopes on CD73 from human mesenchymal stem cells. Biochem Biophys Res Commun. 2001; 289:519–524.
12. Young HE, Steele TA, Bray RA, et al. Human reserve pluripotent mesenchymal stem cells are present in the connective tissues of skeletal muscle and dermis derived from fetal, adult, and geriatric donors. Anat Rec. 2001;264:51–62.
13. Tremain N, Korkko J, Ibberson D, Kopen GC, DiGirolamo C, Phinney DG. MicroSAGE analysis of 2,353 expressed genes in a single cell-derived colony of undifferentiated human mesenchymal stem cells

reveals mRNAs of multiple cell lineages. Stem Cells. 2001;19: 408–418.
14. Reyes M, Dudek A, Jahagirdar B, Koodie L, Marker PH, Verfaillie CM. Origin of endothelial progenitors in human postnatal bone marrow. J Clin Invest. 2002;109:337–346.
15. Reyes M, Lund T, Lenvik T, Aguiar D, Koodie L, Verfaillie CM. Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. Blood. 2001;98:2615–2625.
16. Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation. 2002;105:93–98.
17. Woodbury D, Schwarz EJ, Prockop DJ, Black IB. Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res. 2000;61:364–370.
18. Liechty KW, MacKenzie TC, Shaaban AF, et al. Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep. Nat Med. 2000;6:1282–1286.
19. Mackenzie TC, Flake AW. Human mesenchymal stem cells persist, demonstrate site-specific multipotential differentiation, and are present in sites of wound healing and tissue regeneration after transplantation into fetal sheep. Blood Cells Mol Dis. 2001;27:601–604.
20. Majumdar MK, Thiede MA, Haynesworth SE, Bruder SP, Gerson SL. Human marrow–derived mesenchymal stem cells (MSCs) express hematopoietic cytokines and support long-term hematopoiesis when differentiated toward stromal and osteogenic lineages. J Hematother Stem Cell Res. 2000;9:841–848.
21. Majumdar MK, Thiede MA, Mosca JD, Moorman M, Gerson SL. Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells. J Cell Physiol. 1998;176:57–66.
22. Cheng L, Qasba P, Vanguri P, Thiede MA. Human mesenchymal stem cells support megakaryocyte and pro-platelet formation from CD34+ hematopoietic progenitor cells. J Cell Physiol. 2000;184:58–69.
23. Bartholomew A, Sturgeon C, Siatskas M, et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp Hematol. 2002;30:42–48.
24. Simonsen JL, Rosada C, Serakinci N, et al. Telomerase expression extends the proliferative lifespan and maintains the osteogenic potential of human bone marrow stromal cells. Nat Biotechnol. 2002;20:592–596.
25. Shi S, Gronthos S, Chen S, et al. Bone formation by human postnatal bone marrow stromal stem cells is enhanced by telomerase expression. Nat Biotechnol. 2002;20:587–591.
26. Kawano Y, Kobune M, Yamaguchi M, et al. Ex vivo expansion of human umbilical cord hematopoietic progenitor cells using a coculture system with human telomerase catalytic subunit (hTERT)-transfected human stromal cells. Blood. 2003;101:532–540.
27. Harrington L, Zhou W, McPhail T, et al. Human telomerase contains evolutionarily conserved catalytic and structural subunits. Genes Dev. 1997;11:3109–3115.
28. An International System for Human Cytogenetic Nomenclature (1985) ISCN 1985 . Report of the Standing Committee on Human Cytogenetic Nomenclature. Birth Defects Orig Artic Ser. 1985;21:1–117.
29. Galmiche MC, Koteliansky VE, Briere J, Herve P, Charbord P. Stromal cells from human long-term marrow cultures are mesenchymal cells that differentiate following a vascular smooth muscle differentiation pathway. Blood. 1993;82:66–76.
30. Kobune M, Xu Y, Baum C, Kelley MR, Williams DA. Retrovirus-mediated expression of the base excision repair proteins, formamido-pyrimidine DNA glycosylase or human oxoguanine DNA glycosylase, protects hematopoietic cells from N, N′, N″-triethylenethiophosphoramide (thioTEPA)-induced toxicity in vitro and in vivo. Cancer Res. 2001;61:5116–5125.
31. Deans RJ, Moseley AB. Mesenchymal stem cells: biology and potential clinical uses. Exp Hematol. 2000;28:875–884.

32. Charbord P, Tippens D, Wight TS, Gown AM, Singer JW. Stromal cells from human long-term marrow cultures, but not cultured marrow fibroblasts, phagocytose horse serum constituents: studies with a monoclonal antibody that reacts with a species-specific epitope common to multiple horse serum proteins. Exp Hematol. 1987;15:72–77.

33. Dennis JE, Charbord P. Origin and differentiation of human and murine stroma. Stem Cells. 2002;20:205–214.

34. Loeuillet C, Bernard G, Remy-Martin J, et al. Distinct hematopoietic support by two human stromal cell lines. Exp Hematol. 2001;29:736–745.

35. Morales CP, Holt SE, Ouellette M, et al. Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. Nat Genet. 1999;21:115–118.

36. Bodnar AG, Ouellette M, Frolkis M, et al. Extension of lifespan by introduction of telomerase into normal human cells. Science. 1998;279:349–352.

37. Yang J, Nagavarapu U, Relloma K, et al. Telomerized human microvasculature is functional in vivo. Nat Biotechnol. 2001;19:219–224.

38. Kiyono T, Foster SA, Koop JI, McDougall JK, Galloway DA, Klingelhutz AJ. Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. Nature. 1998;396:84–88.

39. Okamoto T, Aoyama T, Nakayama T, et al. Clonal heterogeneity in differentiation potential of immortalized human mesenchymal stem cells. Biochem Biophys Res Commun. 2002;295:354–361.

40. Bartholomew A, Patil S, Mackay A, et al. Baboon mesenchymal stem cells can be genetically modified to secrete human erythropoietin in vivo. Hum Gene Ther. 2001;12:1527–1541.

41. Devine SM, Hoffman R. Role of mesenchymal stem cells in hematopoietic stem cell transplantation. Curr Opin Hematol. 2000;7:358–363.

42. Bianco P, Riminucci M, Gronthos S, Robey PG. Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells. 2001;19:180–192.

43. Breems DA, Blokland EA, Neben S, Ploemacher RE. Frequency analysis of human primitive haematopoietic stem cell subsets using a cobblestone area forming cell assay. Leukemia. 1994;8:1095–1104.

44. Mobest D, Goan SR, Junghahn I, et al. Differential kinetics of primitive hematopoietic cells assayed in vitro and in vivo during serum-free suspension culture of CD34^{+} blood progenitor cells. Stem Cells. 1999;17:152–161.